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BONE MARROW MALFUNCTION ANEMIA TREATMENT AGENT

(Kotsuzui kino shogai sei hinketsu chiryo zai)

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Specification

1. Title of Invention

BONE MARROW MALFUNCTION ANEMIA TREATMENT AGENT

2. Scope of Patent Claims

1.The bone marrow malfunction anemia treatment agent consists of homoerythropoietin as the effective component.

2.The bone marrow malfunction anemia treatment agent of Claim 1 is characterized in that the homoerythropoietin is obtained from the genes coded with the amino acid arrangement of the human erythropoietin inside the host cell.

3.The bone marrow malfunction anemia treatment agent of Claim 1 is characterized in that the homoerythropoietin originates from human urine.

4.The bone marrow malfunction in the bone marrow malfunction anemia treatment agent stated in Claims 1 to 3 is caused by radiation or by taking cancer treatment drugs or this condition is hereditary.

5.The cancer treatment drugs that causes the bone marrow malfunction in the bone marrow malfunction anemia treatment agent stated in Claim 4 are the cancer suppressing drugs, 1 or more than 2 or these drugs are selected from this list

[1] the numbers in the margin indicate pagination in foreign text

consisting of Cisplatin, 5 - Fluorouracil, Melphalan, Cyclophosphamide, Busulfan, Mesotrexate, Tegafur, Citrabin, Mercaptopurine, Doxorubicin, Acralbisin, Breomycin, Peplomycin, Mytomycin C, Actinomycin D, Pinclistine, ethopoxide.

6.The drug to suppress the side effects of the cancer suppressing drug consists of homoerythropoietin as the effective component.

7.The cancer suppressing drugs used in suppressing the side effects of the cancer suppressing drug of Claim 6 are the cancer suppressing drugs shown in Claim 5.

3. Detailed explanation of the invention

[Industrial field of use]

The invention pertains to a bone marrow malfunction anemia treatment agent containing homoerythropoietin as the effective component.

[Prior Art]

Anemia is the condition when the amount of iron in the blood is below that of a normal person. This condition is as a result of many causes. /2

One cause of anemia is that the blood production is obstructed and this obstruction is due to hereditary factor or it is as a result of taking cancer suppressing drugs or medicine and from radiation (Depart of hematology, Takaku et al (2nd edition), University Hospital, pages 14 to page 24).

Conventionally, the treatment method for this type of bone marrow malfunction anemia is to administer male hormones, for example, Nandrolone, Phepropionate, Mestanolone, Nandrolone phenyl propionate. However, when the symptoms do not improved from the administration of these male hormones, the last resort is to perform a bone marrow transplant.

[The problems resolved by the invention]

However, there are a lot of side effects from using these protein production hormone drugs, such as, liver malfunction, menstruation abnormality, etc. Also, there are problems like infection. Also, in addition to the side effects to the liver organ from taking the male hormones, there are side effects on both male and female patients. (refer to Osaka Prefecture Hospital Pharmaceutical Society Edition, Pharmaceutical Guideline (the 4th Edition), the Professional Edition, 1977, p662 - p699). In addition, during the bone marrow transplant, there are problems with autoimmune disorder after the transplant, infection, etc. Also, it is difficult to even treat with very aggressive treatment methods (refer to Clinical Treatment, 15(9): 687 - 699, 1983).

The purpose of the invention is to focus on the problems to treat patients with this bone marrow malfunction anemia and to offer an excellent treatment agent that has more safety.

[Means for resolving the problems]

As a result of focusing their earnest research efforts for achieving the above purpose, the inventors discovered an effective drug for the treatment of bone marrow malfunction anemia. The effective component of this treatment drug is erythropoietin (refer to below as EPO).

That is, the invention offers a bone marrow malfunction anemia treatment agent consisting of homoerythropoietin as the effective component. Also, since the agent of the invention is effective in reducing the side effects of the cancer suppressing drug to treat the bone marrow malfunction. Based on this, it is also offered as the side effect reducing agent for the cancer suppressing drug which contains homoerythropoietin as the effective component.

The invention is explained in detail below.

Anemia is the condition when the amount of iron in the blood is below that of a normal person. This condition is as a result of many causes. One cause of anemia is that the blood production is obstructed and this obstruction is due to a hereditary factor or it is as a result of taking cancer suppressing drugs or medicine and from radiation.

Furthermore, the examples of the cancer suppressing drugs that causes the aforementioned bone marrow malfunction anemia are such Cisplatin, 5 - Fluorouracil, Melphalan, Cyclophosphamide, Busulfan, Methotrexate, Tegafur, Citrabin,

Mercaptopurine, Doxorubicin, Acralbisin, Breomycin, Peplomycin, Mytomycin C, Actinomycin D, Pinclistine, ethopoxide. Also, the agent of the invention is effective as the side effect reducing agent for the side effects resulting from taking these drugs.

EPO is not limited to the human kind but it is present in red blood cells that exists in the bone marrow of various types of animals. Microbiology displays the action of promoting the propagation and growth of these in the red blood cells. The human EPO used in the invention is a polypeptide having the amino acid arrangement that is one characteristic in human, it consists of or does not consists of a suitable sugar chain. Specifically, it originates from human urine. It is obtained from the gene coded with the amino acid arrangement of the human EPO inside the host cell (refer to below as human rEPO). It is obtained by culturing the hybrimer obtained from cell fusion of the original human cell group having EPO production capability or it is obtained from the culture of human cells.

Human EPO used in the invention can be obtained by various means.

For example, the human urine EPO can be obtained by extracting from the blood (containing the blood serum) or the urine of an anemic human subject and from a patient with reoccurring anemia (T. HIYAKE et al, Journal of Biological Chemistry - (J.B.C), Vol. 252, p. 5558 (1977); J. P. Lewin et

al, Journal of Laboratory and Clinical Medicine (J. Lab. Clin. Med.), Volume 66, p. 987 (1965)). /3

Also, the messenger - RNA (mRNA) corresponding to human rEPO, for example, the amino acid arrangement of the human EPO is collected. The substitution DNA is produced using that mRNA. Next, it is produced with a suitable host cells (for example, the cells of plant or animals, the enzymes, the bacteria species like that of the large intestines), it is obtained according to a genetic engineering method. (For example, refer to SYLVIA L. H. et al: Proceedings of the National Academy of Science U.S.A. (Proc. Natl. Acad. Sci. USA) Vol. 81, page 2708 (1984)).

Various cells can be used but it is preferred that the aforementioned animals cells used are the cell culture from human or mammals. For example, the COS cells, the Chinese Hamster ovaries (CHO) cells and the mouse C - 127 cells can be used. In addition, the methods used are the method using the tissue culture from human kidney cells (Patent Publication No. 54 - 55790), the method using the lymphocyte cells from human having the homoEPO production capability (Patent Publication No. 57 - 40411) and the method from culturing the hybrid obtained by cell fusion of the human cell group.

The human EPO obtained according to the invention is effective in the treatment of bone marrow malfunction anemia,

the mature red blood cells that are propagated have the capability to carry sufficient oxygen.

In the above described methods, the human EPO contained in the urine or the culture skim can be made concentrated and purified by the separation and purification method if desired. For example, the sedimentation method according to the organic solvent such as benzoic acid, ethanol, acetone and tannic acid; various types of chromatography are performed such as gel chromatography, ionic exchange chromatography, affinity chromatography; the electrophoresis method such as the equal potential electrophoresis swim and the gel electrophoresis. These methods can be used alone but it is preferred that all these methods are combined together.

The human EPO can be stored by removing its moisture by the means of freezing or freeze drying or vacuum dry. In addition, the effective component is precipitated by adding a water affinity organic solvent or a water soluble salt in the human EPO containing an aqueous solution. The sediment that is obtained is dried and then put into storage. Also, if desired, the human EPO is dissolved in a suitable buffer solution, then, it is filtered with a millipore filter and used in injection.

The bone marrow malfunction anemia treatment agent of the invention can be mixed with other components during use or mixed with an iron tablet, a vitamin B12 tablet or a male hormone. The

examples of the iron tablet used are such as dry iron (I) sulfate, iron fumarate, iron dextrane, iron gluconate, iron glucoronate and iron orotane.

The dosage and frequency of the administration of human EPO contained in the bone marrow malfunction anemia treatment agent of the invention are determined according to the condition in the patients. However, typically, the human EPO is administered once a day at 500 - 100000 U/day per adult for at least 2 weeks.

Also, it can be administered as a shot by the normal intravenous injection or subcutaneous injection.

It is preferred that the bone marrow malfunction anemia treatment agent of the invention contained a stabilizing agent. The examples of the said stabilizing agent are such as polyethylene glycol, protein, sugar, amino acid, inorganic acid, organic acid and a sulfate containing a reducing agent.

It is preferred that the addition amount of these stabilizing agents are combined at a proportion of 0.11 - 1000 wt. pts. to 1 wt. pt. Of the human EPO. Furthermore, when more than 2 stabilizing agents are combined, the total amount must be within the above prescribed range.

These stabilizing agents can be used at an amount where the pH and the temperature of the aqueous solution can be adjusted. The permeation pressure ratio of this aqueous solution is in the range of 0.1 - 3.0 but more preferably in the range of 0.8 -

1.2. The pH of the aqueous solution is adjusted to 5.0 - 9.0. In particular, it is preferred that the pH is adjusted to 6 - 8.

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Also, it is preferred that the absorption prevention agent is added when adjusting the contents of the agent of the invention.

[Implementation example]

The invention is explained specifically with the participation examples (human EPO production example), the experiment examples (the efficacy of the drug) and the implementation examples (the preparation of the drug).

Participation example 1 Production of human urine EPO

Step 1) partial purification from human urine

The partial purified human urine EPO is obtained by using 1) The salt removal by Sephadex G50, 2) The Patch absorption by DEAE cellulose, 3) The ethanol sedimentation, 4) The DEAE agalose column chromatography from the urine of a patient with re-occurring anemia, the method is performed according to the method by HIYAKE T. et al (Journal of Biological Chemistry - (J.B.C.) Vol. 52, page 5558 (1977 year).

Step 2) Reverse phase chromatography

The purification is performed by HPLC, the partially purified human urine EPO that is obtained is dissolved in 0.1 % of trifluoro acetic acid (made by Aldrich Co.) containing 24 %

of propanol (made by Wako Pure Chemicals). The HPLC device is the 638 - 50 model made by Hitachi, the detection is performed by the ultraviolet ray absorption of 280 nm and 220 nm.

The specimen obtained is injected into the YMC - C8 column (6 mm x 30 cm, made by Yamamura Chemical Co.) that is previously equalized with 0.1 % trifluoro acetic acid solution containing 24 % of n - propanol. After the unabsorbed portion is eluted, the concentration of the n - propanol is increased to 26 % and eluted. After the active portion of the EPO is collected, it is removed by filtration using Centricon - 100 (made by Amicon Co.) and concentrated to 0.1 - 0.2 ml.

Step 3 High speed molecular chromatography

The concentrated specimen obtained from above is poured into TSK - G 300 SW column (7.8 mm x 60 cm, made by Toyotatsu Co.) that has been previously equalized with 0.1 % TFA solution containing 26 % of n - propanol, it is eluted with the aforementioned equilibrium solution. The peak containing the EPO activity at the position having the molecular weight in the range of 25000 - 30000 is collected and freeze dry. The specific activity is about 9 x 10 power of 4 U/mg.

The specific activity is shown in Table I in each step.

Table I

Step	Specific activity x (U/mg)
1) Partial purification	600

2) Reverse phase chromatography 10000

3) High speed molecular chromatography 90000

* Assay method - According to the Iscove N. N et al method (Journal of cellular Physiology), Vol 83, page 309 (1974))

Participation example 2 Production of Human r EPO that originates from CHO cells.

According to the method disclosed in (Patent Publication No. 59 - 281862), the title of the invention applied in December 27 is "Vector containing the supplementary DNA for the phenotypic expression of the nucleus cells", the human rEPO is obtained by the phenotypic expression with the plasmide containing the genes that are coded with the amino acid arrangement of the homoEPO from the Chinese hamster - ovaries cells (CHO cells). The outline for this is given below.

The DNA from the lamda HEOPEL13 crone combined with the genes that coded with the amino acid arrangement of the human EPO obtained from the human embryonic liver cells are digested in EcoR1. The small R1 fragments containing the genes coded with the amino acid arrangement of human EPO are extracted and inserted into the 1 part of the EcoR1 of the plasmide RKI - 4. The plasmide RKI - 4 combined with theses human EPO genes are phenotypically transformed into the DHFR - defective CHO. The cells are selected, these cells have at least one DHFR gene by cultivating in a culture medium of alpha where the CHO cells are

lacking in nucleic acid. The human r EPO is produced by increasing gradually the concentration of the Methotrexate. The activity of the human r EPO on the top skim of the final culture is at 20 U/ml.

After the CHO cells are cultured for 3 days in the non-blood serum culture medium, the human rEPO is prepared according to the purification method using the human urine EPO. The human rEPO that is obtained has an activity level of 6600 U/ml according to the Krystal et al Method (Journal of laboratory Clinical Medicine (J. Lab. Clin. Med.) vol. 97, page 144 (1981)). Also, this human rEPO is recognized with a single band from the result of the SDS polyacryl amide gel electrophoresis. /5

0.1 % of BSA is added to the human eEPO that is obtained. After dissolving in the physiological saline solution, it is used in the experiment.

Experiment example 1

The anemia improvement effect of human EPO on the mouse suffering from the bone marrow malfunction anemia due to defective genes

1. Experiment animal:

The W type mouse showing the genetically defective bone marrow malfunction anemia is used. Also, the anemia sickness in this mouse is known to resemble the re-occurring jaundice sickness. Specifically, these groups are used, the (WB x

C57BL/6) F1 - W/Wv group, -Wv/+group and the -+/+ group of mouse (9 weeks old, weight = 21 - 34 g) used as comparison, these are treated for 1 week and used as comparison.

2. EPO: The human rEPO originate from the CHO - cell is used. The EPO according to the administration amount is dissolved in an aqueous solution containing 0.05% of human blood serum albumin.

3. The experiment method:

The administration of the human rEPO is implemented for 7 days via intravenous administration from the back of the mouse. Before administration, the weight of each group of mouse is weighed. The administration amount of the human rEPO is 0, 17, 86, 8600 U/Kg are used as comparison examples. The administration amount of the specimen is 5 ml/kg. The measurement is performed at the final administration day. The collection of the measured sample is performed by collecting blood rapidly from a large vein applied with ether anesthetics.

4. Result:

The result is shown in Table 2. According to Table 2, the hemoglobin amount (g/dl) in the blood shows an increase due to the administration of the human rEPO 86 U/kg in the Wv/+ group of mouse and a significant increase due to the human rEPO 8,600 U/kg administration in the W/Wv group of mouse. The anemia improvement effect is exhibited.

Table - 2

Groups; N; Amount used U/kg; hemoglobin g/dl

表 - 2

系統	N	用量U/kg	ヘモグロビンg/dl
+/+	5	0	14.06 ± 0.12
	5	86	15.14 ± 0.30 **
W^V/+	5	0	13.68 ± 0.24
	5	17	13.56 ± 0.51
	5	86	14.84 ± 0.22 **
W/W^V	7	0	10.09 ± 1.06
	7	86	10.89 ± 0.98
	7	8600	13.47 ± 0.96 *

The average +/- standard deviation difference: $p < 0.05$ **; $P < 0.01$

To the comparison group (human r EPO OU/kg)

Implementation example 2

The anemia improvement effect from the human EPO for the mouse that has received X-ray and bone marrow transplant

1. Experiment mouse:

The mouse that is used is the C57BI/6NCrj.o8W type. This mouse is completely radiated with 850 rad of X ray. Then, the bone marrow nucleus cell of 5 x 10 power of 5 of the same type of mouse is used for the transplant, the X ray radiation and the bone marrow transplant.

2. The preparation of the administration specimen

The human rEPO uses the Lot R6 EO2. This human rEPO is diluted to 96 U/ml with 0.05 % human blood serum albumin and 5% of mannitol solution. The human rEPO is used as the specimen. The human rEPO specimen is divided, freeze dried for storage and it is thawed out on the administration day. The control specimen that does not contain the human rEPO is freeze dried in 5 % of mannitol solution and 0.05 % of human blood serum albumin.

3. The experiment method:

The human rEPO specimen and the control specimen in the freeze dried storage state are thawed out on the day of the administration. These administration specimens are administered at 0.1 ml subcutaneously for a continuous period of 14 days, once a day from the day it received X ray radiation and it undergoes a bone marrow transplant.

The hemoglobin value in the blood serum of the 4 mice are extracted from the non-processed group, the control administration group, the human rEPO administration group at day 1, 4, 7, 14, 21 days (the non-processed mouse, n = 4). /6

The blood collection is performed intravenous into a hematoclit tube, the hemoglobin value is measured with the MICRO CELLCOUNTER CC180A (made by SYSMEX Co.).

4. Result:

When the human rEPO is administered for a continuous period of 14 days to the mouse that has gone through X ray radiation

and bone marrow transplant, the shift in the hemoglobin value is shown in figure 1. As shown in figure 1, the hemoglobin value is reduced when the blood production is stopped but it shows that the red blood cells production is increased due to the recovery. In particular, at day 21, the group that has been administered the human rEPO shows an significant increase of red blood cells, the promotion of the red blood cells production due to the human rEPO is recognized. The anemia condition is improved effectively.

Implementation example 3

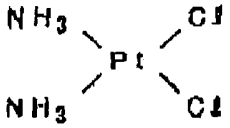
The improvement effect of human EPO on anemia caused by Cisplatin

1. Experiment animal:

A male SD rat (Laboratory animal) weighing 140 - 160 g is used. A group of 4 - 5 rats are used, it is fed freely with food and water.

2. Medicine

Cisplatin (CDDP) (made by Aldrich) is dissolved in physiological saline solution. 1 ml per 100 g of the weight of the rat is injected intravenously. Furthermore, the CCDP structure is given below.



After diluting the human rEPO that originates from CHO - cells with a dilute solution of (5% of mannitol and 0.05 % human blood serum albumin), 0.5 ml is administered intravenously per 100 g weight of the rat.

3. The experiment method:

8 mg/kg of the CDDP is administered once to the rat. The blood is examined with the method shown below at day 13. The hemoglobin becomes uniform. The administration of human rEPO begins from day 13 for a total of 10 administration for once each day. The processing of the solvent (vehicle) is not performed for the group that is not administered with the human rEPO. Human rEPO is administered to the normal rat (the group that is not administered the CDDP)

(The blood examination)

After the CDDP administration, after a time has elapsed, about 20 microliter of blood is collected from the vein in the back using a pipette. The hemoglobin is measured with an automatic blood platelet counting machine (Sysmex CC - 180A).

4. Result

The results are shown in Figure 2. According to the figure, the administration of the human rEPO is changed from 22 U/kg to 170 U/kg, the anemia caused by the CDDP shows improvement depending on the amount of medicine administered.

Experiment example 4

The improvement effect of human EPO on the anemia condition caused by 5 - FU

1. Experiment animal

Male SD rats weighing between 150 - 160 g (laboratory animals) are used. A group of 6 are used and are fed freely with food and water.

2. Medicine:

150 mg/kg of 5-FU is used similar to Experiment example 3.

3. Experiment method:

5-FU of 150 mg/kg is injected into the rat. On the other hand, the hemoglobin from administering the said 5 - FU 8 days before becomes the lowest. The human rEPO is injected intravenously to day 12 for a total of 11 times, a schedule is used for this. The dosage of the human rEPO is administered at 170, 860, 1730, 3460 U/kg. The blood is collected intravenously on the back after an elapsed time, the hemoglobin value is measured.

4. Result

The result is shown in figure 3. It is clear from the diagram that 5 - FU at 150 mg/kg is administered intravenously. 1 week after the administration, anemia appears and at day 12 or more, recovery is displayed (the group that have been administered 5-FU).

On the other hand, in the group using the combination of 5 - FU and the human rEPO, the hemoglobin is increased to above

the normal level before the onset of anemia (4 days before) due to the previous administration of the human rEPO. Anemia appears at more than 8 days. The hemoglobin is reduced with the group that has a combined use of human rEPO. The anemia improvement effect is recognized from this group. The hemoglobin level is held at a normal level when the human rEPO is administered at above 1730 U/kg.

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Implementation example 1

Human rEPO originate from CHO cell	1 wt. pts.
Human blood serum albumin	100 wt. pts.
Total amount of distilled water used for injection	100000 wt.pts.

The solution that is sterilized is prepared with the above proportions. This is stored in a vial, freeze dried and sealed.

Implementation example 2

100 wt. pts. of dextran 40 is used instead of the human blood serum albumin used in Implementation example 1. Similarly, the freeze dried process is performed.

Implementation example 3

A 100 ml of aqueous solution that is sterilized is prepared and this consists of 5 g of mannitol, 1 mg of human urea EPO, 100 mg of human blood serum albumin, 2.154 mg of acetyl triptophan sodium and 1.33 mg of capryl acid sodium. This

mixture is poured into a 1 ml vials each, these are freeze dried and sealed.

Implementation example 4

A 100 ml of an aqueous solution that is sterilized is prepared, it is a 0.05 M of phosphoric acid buffer solution at pH7.0 and this consists of 500 mg of polyethylene glycol 4000, 1 mg of human urea EPO, 30 mg of ethylene oxide propylene oxide copolymer and 800 mg of sodium chloride. This mixture is poured into a 1 ml vials each, these are freeze dried and sealed.

Implementation example 5

A 50 ml of an aqueous solution that is sterilized is prepared, it is a 0.05 M of phosphoric acid buffer solution at pH7.0 and this consists of 1 g of sorbitol, 1g of glycine and 0.5 mg of human rEPO originating from CHO cells. This mixture is poured into a 0.5 ml vials each, these are freeze dried and sealed. Separately, a 0.1 % of methyl cellulose aqueous solution is prepared and sterilized, and poured into 1 ml vials each, it is used as the solvent.

Implementation example 6

100 ml of an aqueous solution is prepared and sterilized, this consists of 1 mg of human urea EPO, 500 mg of human blood serum albumin and 500 mg of mannitol. These are poured into a 1 ml vials each and freeze dried, then sealed.

Separately, 300 ml of an aqueous solution is prepared and sterilized, this consists of 3g of iron II gluconate and 2.7 g of NACl, these are poured into 3 ml vial each and sealed. Each of the vials are dissolved gradually in 1 ampule (at 2 - 3 minutes).

4. Brief explanation of the diagrams

Figure 1 is the diagram showing the effect of the human rEPO administration for treating the mouse that has undergone X ray radiation and bone marrow transplant. Figure 2 is the diagram showing the effect of the human rEPO administration for treating the mouse with anemia caused by CDDP. Figure 3 is the diagram showing the effect of the human rEPO for treating the mouse with anemia caused by 5-FU.

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Figure 1

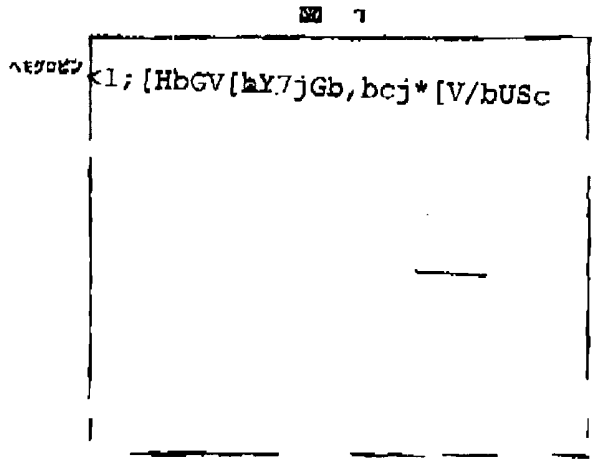

Figure 2